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CRYOPRESERVATION OF SHOOT TIPS OF BLACKBERRY AND RASPBERRY BY ENCAPSULATION-DEHYDRATION AND VITRIFICATION

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Abstract

Encapsulation-dehydration and PVS2-vitrification cryopreservation protocols were evaluated for the long-term conservation of a diverse group of *Rubus* germplasm. Cold acclimation for a 4-week period prior to cryopreservation was necessary for regrowth of shoot apices from blackberry and raspberry genotypes. For the encapsulation-dehydration protocol, encapsulated apices were pretreated in 0.75 M sucrose for 20 h, desiccated 6-h under laminar flow to ~20% moisture content, then plunged in liquid nitrogen (LN) and rapidly warmed. The PVS2-vitrification protocol included pretreating shoot tips on 5% dimethyl sulfoxide (DMSO) medium for 48 h, exposure to loading solution (LS) and PVS2 for 20 min each at 25ºC, followed by immersion in LN and rapid warming. Shoot tips of 25 genotypes in 9 *Rubus* species and 9 *Rubus* hybrids were successfully cryopreserved with recovery of 60-100% using the encapsulation-dehydration protocol. Four genotypes of 3 species were tested using the vitrification protocol with 71% average regrowth. The present results indicate that both of these improved cryopreservation protocols can be applied to a diverse range of *Rubus* genetic resources.

Keywords: cold acclimation, germplasm, liquid nitrogen, *Rubus*

INTRODUCTION

Rubus L. of the family Rosaceae is an economically and ecologically important genus with 12 subgenera and about 750 species. It displays a high degree of morphological diversity (1). The two large subgenera are *Eubatus* (blackberries, 2 to 7x) and *Idaeobatus* (raspberries, predominantly diploid). *Rubus* is an important crop in the north-temperate zone in Europe and North America. Though considered a minor fruit crop in India, *Rubus* has much diversity: 27 species grow wild in the Indian Himalayan region (27). A large collection of *Rubus* germplasm is maintained at National Clonal Germplasm Repository (NCGR), Corvallis, Oregon, USA, in the form of potted plants in greenhouse/screenhouse and *in vitro*, and at National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India, in the field

genebanks and *in vitro*. The preservation of clonal germplasm is problematic, since whole plants or their tissues need to be conserved (3).

Among the currently available methods for long-term *ex situ* conservation of clonally propagated germplasm, cryopreservation of shoot apices is the most reliable, cost- and space-effective option (10, 22). Vitrification-based procedures such as PVS2-vitrification (VIT) and encapsulation-dehydration (E-D) are gaining importance and have greater potential for broad applicability. Vitrification involves the application of cryoprotectant solutions that increase cell viscosity to a critical point at which water forms a meta-stable glass on exposure to ultra-low temperatures. Encapsulation-dehydration involves removing cell water through osmotic and evaporative dehydration to achieve the same state. Cryopreservation is increasingly used to conserve crop plant germplasm (2) including various herbaceous and woody plant species of temperate (20) and tropical (26) regions. Cryopreservation of dormant *Rubus* floral buds in LN was reported by Gu et al. (11); *in vitro* shoot tips of the same species were cryopreserved by slow freezing with 60 to 100% regrowth (6, 17, 19). Recently, encapsulation-vitrification and encapsulation-dehydration were compared for *Rubus idaeus* L. cultivars (29). For the seven raspberries tested encapsulation-vitrification was the most successful protocol with 75% regrowth compared to 50% for a modified encapsulation-dehydration protocol (29). The strategy of the present study was to develop a vitrification protocol for cryopreservation of a wide range of *Rubus* genotypes using encapsulation-dehydration and PVS2-vitrification. We report the successful cryopreservation of shoot apices of 25 genotypes of blackberry and raspberry cultivars using encapsulation-dehydration and vitrification techniques.

MATERIALS AND METHODS

Plant material

Micropropagated plantlets of 25 genotypes of 9 *Rubus* species were studied (Table 1). Six genotypes were used to develop protocols and the remaining genotypes were used to test protocol efficacy. *In vitro* plants were multiplied on NCGR-*Rubus* (RUB) medium which contains MS (15) mineral salts and vitamins with double EDTA-Fe, 2 mg/l N^6 benzyladenine (BA), 0.1 mg/l indole 3 butyric acid (IBA), 0.1 mg/l GA_3 (Sigma-Aldrich Co., St. Louis, MO), 3.5 g/l agar (Difco, Detroit, MI), 1.45 g/l gelrite (Phyto Technology Lab, Shawnee Mission, KS) and 30 g/l sucrose at pH 5.7 in Magenta GA_7 boxes (Magenta Corp., Chicago, IL). The plantlets were subcultured every 3 weeks, and grown at 25ºC with a 16 h light/8 h dark photoperiod (40 $\mu E.m^{-2}.s^{-1}$).

Cold acclimation

Plantlets were cold acclimated 3 weeks after the last subculture (22ºC with 8 h light (10 $\mu E.m^{-2}.s^{-1}$)/-1ºC 16 h dark) for 1, 2, 3 or 4 weeks. Shoot tips with 2 to 3 pairs of leaf primordia (about 1 mm long) were excised from cold acclimated shoots for all the experiments.

Encapsulation-dehydration procedure

The method developed for pear (9) was used with some modifications. Excised shoot tips were encapsulated in alginate beads composed of 3% (w/v) low viscosity alginic acid (Sigma Chemical Co. USA) in liquid MS medium without calcium, pH 5.7 and allowed to polymerize for 20 min in MS medium with 100 mM $CaCl_2$ and 0.4 M sucrose. Beads were pretreated in liquid MS medium with 0.75 M sucrose for 20 h on a rotary shaker (50 rpm). Beads were then blotted dry on sterile filter paper and desiccated for 6 h [approx. 20% moisture content] in a glass Petri dish under laminar flow (0.6 m/sec) at ambient temperature of ~25ºC and at 35 ± 2 % relative humidity (%RH). Dried beads were placed in 1.2 ml cryovials (10 beads/cryovial)

and plunged directly into liquid nitrogen (LN). The vials were rewarmed in 45ºC water for 1 min and in 25ºC water for 2 min. The beads were rehydrated in liquid MS medium for 5 min and transferred onto recovery medium (RUB with no IBA) in 24-cell plates for regrowth, one shoot tip per cell (Costar, Cambridge, Mass.). To determine the effect of each stage of the process on the regrowth of shoot tips following cryopreservation, we plated a subset of shoot tips at each critical step of the procedure: (1) after encapsulation of shoot tip in alginate bead (encapsulation), (2) after 20 h treatment in liquid 0.75M sucrose medium (osmotic dehydration), (3) after evaporative dehydration under laminar flow (air dehydration), and (4) after LN exposure (LN).

Air desiccation

Shoot tips excised from 4-week cold acclimated plants of 'Burbank Thornless' were encapsulated and pretreated as above. Beads were desiccated under laminar flow for 2, 4, 6 and 8 h, plunged into LN for 1 h, rewarmed and plated as described above. Three sets of 20 desiccated beads were used to determine (1) moisture content, (2) regrowth after air dehydration, and (3) regrowth after air dehydration and LN exposure. Three replicates of 25 beads (n=75) were tested for moisture content for each of the 3 treatments. The beads were weighed and dried at 103 ± 2ºC for 17 ± 1 h. Moisture content (MC%) was calculated by [(Fresh Weight-Dry Weight)/Fresh Weight] x 100 (13). 'Fresh weight' represents the weight of beads before oven drying but after all pretreatments (i.e. step 1 to 3). Residual moisture (RM %) was calculated by [(Fresh Weight-Dry Weight)/Dry Weight] x 100 and presented as g water/g dry weight (5).

Vitrification procedure

The PVS2-vitrification protocol developed for white clover (30) was modified for this study. Excised shoot tips were pretreated for 48 h on MS medium containing 5% dimethyl sulfoxide [DMSO (Sigma-Aldrich Co., St. Louis, MO)] (v/v) with 3.5 g agar and 1.75 g/l gelrite under the standard cold-acclimation conditions. Shoot tips were transferred into 1.2 ml cryo-vials and treated with 1 ml loading solution (LS) (2M glycerol in 0.4 M sucrose solution) for 20 min at either 0ºC or 25ºC. LS was removed and PVS2 cryoprotectant (25) solution [(v/v) 30% glycerol, 15% ethylene glycol and 15% DMSO in liquid MS medium with 0.4 M sucrose at pH 5.8] was added into cryovials for 15, 20, 30 or 40 min at either 0ºC or 25ºC. After PVS2 treatment, vials were submerged in LN for 1 h. Rewarming was as described above. The shoot tips were immediately rinsed twice in liquid MS medium containing 1.2 M sucrose. Shoot tips were planted on recovery medium as described above.

Regrowth assessment & data analysis

Data on regrowth were recorded 4 weeks after rewarming and presented as means and standard deviations (SD). Callus production was noted. Data were subjected to Analysis of Variance and Fisher's Least-Significant-Difference Test using SYSTAT 8.0 software (28). Significance is stated at $P \leq 0.05$. Twenty shoot tips per LN treatment and 5 shoot tips for each pretreatment control were used per replicate. Each treatment was done three times.

Table 1: *Rubus* genotypes cryopreserved in this study.

Taxon / Cultivar	USDA-Plant Introduction No.	NCGR-Local No.
Blackberry		
R. caesius L. (wild)	PI 324058	28.001
R. caucasicus Focke seedling selection	PI 553143	54.001
R. cissoides Cunn. Bush Lawyer (wild selection)	PI 553163	772.001
R. drejeri G. Jensen ex Lange (wild)	PI 553186	55.002
R. hybrid cv. Ebony King	PI 553251	73.001
R. hybrid cv. Olallie (Black Logan x Young 6x)	PI 553255	76.001
R. hybrid cv. Loch Ness (complex hybrid derived from Comanche, Chehalem, early Harvest, Thornfree and selection from Illinois and North Carolina 4x thornless arching)	PI 638182	1863.001
R. hybrid cv. Cherokee (Darrow x Brazos)	PI 553247	67.001
R. hybrid cv. Black Diamond (Kotata x NZ 8610L-163 (E90 x n-71)	PI 638257	2229.001
R. hybrid (Olallie x OSC 878) OSC & USDA selection	PI 553281	344.002
R. hybrid cv. Chehalem (Santiam x Himalaya 6x)	PI 553273	761.001
R. hybrid cv. Ashton Cross (selection of wild European blackberry 4x thorny)	PI 553278	317.001
R. hybrid cv. Chester Thornless (SIUS 47 x Thornfree)	PI 553322	839.001
R. hybrid cv. Hull Thornless (SIUS 47 x Thornfree)	PI 553299	389.001
R. hybrid cv. Dirksen Thornless (US 1482 x Darrow) x Thornfree 4x	PI 553251	71.001
R. ursinus Cham. and Schldl. cv. Burbank Thornless	PI 554060	250.001
R. ursinus Cham. and Schldl. cv. Santiam	PI 554062	79.001
Raspberry		
R. crataegifolius Bunge cv. Jingu Juegal	PI 553178	267.001
R. spectabilis Pursh Olympic Salmonberry (wild selection)	PI 553988	435.001
Black raspberry		
R. occidentalis L. cv. Munger	PI 553740	490.001
Red raspberry		
R. idaeus L. cv. Norfolk Giant	PI 618401	1814.001
R. idaeus L. cv. Skeena	PI 553374	124.001
R. idaeus L. cv. Washington	PI 553380	130.001
R. idaeus L. cv. Willamette	PI 553362	386.001
R. idaeus L. cv. Comet (Ottawa x Madawaska)	PI 553556	1165.001

RESULTS

Encapsulation-dehydration

Cold acclimation: Regrowth of encapsulated and osmotically-dehydrated shoot tips was not affected by the duration of cold acclimation (Fig.1). Shoot recovery of air-desiccated apices, as well as that of LN treated apices, improved significantly ($P \leq 0.05$) with increasing cold acclimation. The maximum shoot regrowth after LN (85%) was reached after 4 weeks of cold acclimation. There was no regrowth following LN exposure with 1 week cold acclimation. Thus, for further experiments plants were cold acclimated for 4 weeks. The plants did not produce callus at any stage of recovery.

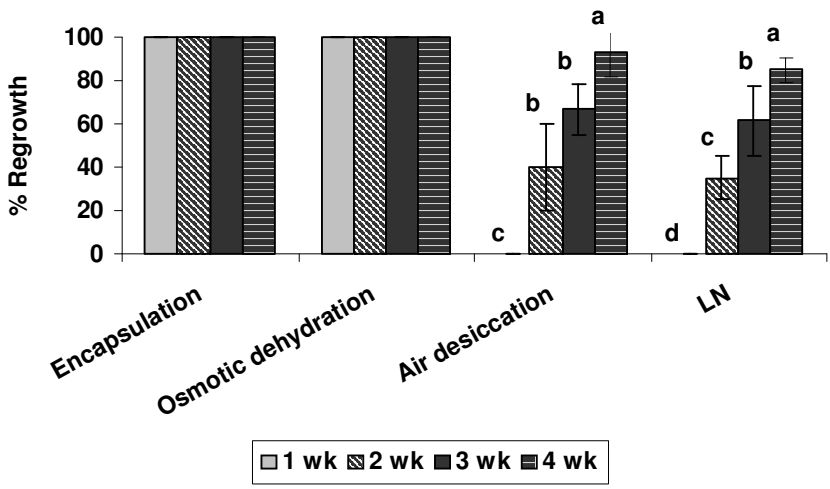

Figure 1: Regrowth of shoot apices of 1-4 wk cold acclimated *R. ursinus* cv. Burbank Thornless after encapsulation-dehydration and LN exposure. Encapsulated apices were air desiccated for 6 h and plunged in LN. Data are means ± SD. Means in a treatment category followed by different letters are significantly different at $P \leq 0.05$.

Air desiccation: Alginate beads initially contained RM of about 3.14 g water/g dry wt (77% MC). Within 2 h of desiccation RM declined to 0.51 and by 6 h to 0.26 (Table 2). Shoot tips of 'Burbank Thornless' with 20.7% to 35% moisture content exhibited about 80% regrowth but viability declined sharply with further desiccation (Fig. 2A). Regrowth of cryopreserved shoot tips was not significantly different between 4 h (86.6% regrowth; 29% MC) and 6 h (93% regrowth; 20.7% MC) air-desiccation times and between 6 h and 8 h (55% regrowth; 19% MC) ($P \leq 0.05$) (Fig. 2B). The regression analysis showed that the beads dried as a linear function over time but regrowth was a quadratic function. The optimal dehydration was 5 h and 22% moisture (Fig. 2B).

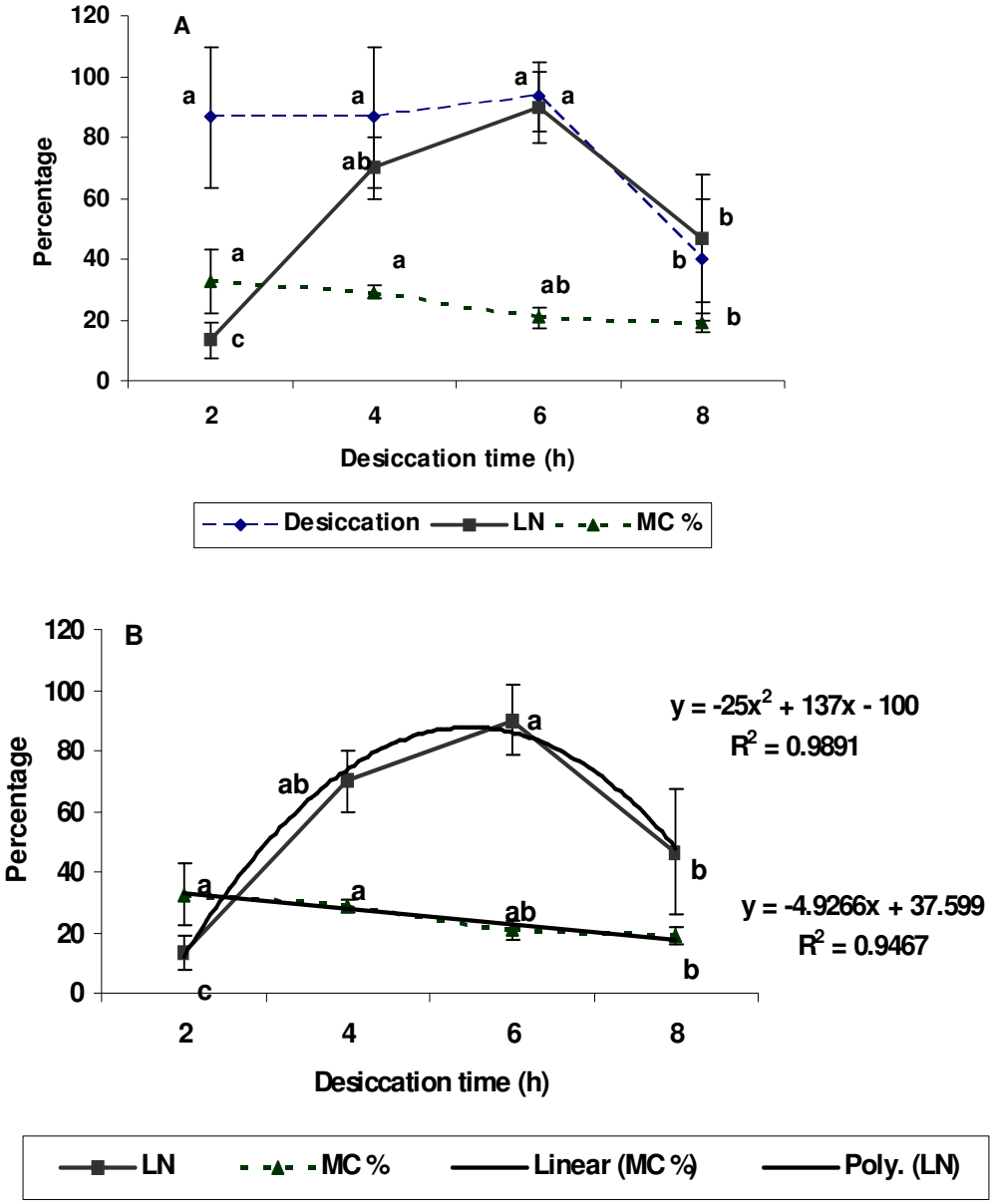

Figure 2: A) Regrowth of shoot apices of *R. ursinus* cv. Burbank Thornless exposed to LN following the encapsulation-dehydration protocol as affected by percent moisture content (MC) of beads. Encapsulated apices were air desiccated for 2, 4, 6 and 8 h and plunged in LN. Data are means ± SD. B) Regression analysis. Means in a category followed by different letters are significantly different at $P \leq 0.05$.

Table 2: Water content of alginate beads (fresh weight and dry weight basis) after air desiccation of beads for 0, 2, 4, 6 and 8 h. Data are means ± SD (n=75).

Time (h)	Moisture content % (fresh wt basis)	g water/ g dry wt
0	77.26 ± 4.19	3.14 ± 0.01
2	32.52 ±1.27	0.51 ± 0.24
4	29.0 ± 2.16	0.41 ± 0.04
6	20.74 ± 3.41	0.26 ± 0.05
8	18.85 ± 3.0	0.25 ± 0.04

Genotype response: All genotypes tested responded very well to the encapsulation-dehydration protocol (Fig. 3). There were no significant differences among the genotypes by fruit type however raspberries 'Norfolk Giant' and *R. spectabilis* 'Olympic Salmonberry' had significantly greater recovery than 'Burbank Thornless' and *R. crataegifolius* 'Jingu Juegal.' There were no significant differences among the other cultivars. Leaves elongated within 5 to 7 days after rewarming. New shoots emerged from the rehydrated beads in 2 to 3 weeks without intermediary callus. The growth pattern of LN-exposed shoot tips was not different from those of controls.

Efficacy of encapsulation-dehydration protocol: The encapsulation-dehydration protocol was further tested on 18 genotypes (Table 3). The encapsulation and osmotic dehydration steps did not affect viability, but up to 20% viability was lost following air desiccation. The viability of some genotypes was further reduced by LN exposure. Regrowth following LN ranged from 60% to 100%. The protocol worked successfully for both blackberry and raspberry cultivars. Only 'Loch Ness', 'Skeena' and 'Munger' had recovery < 80% following LN exposure.

Table 3: Regrowth of shoot apices of 18 *Rubus* genotypes cryopreserved using the encapsulation-dehydration protocol with 6 h desiccation and rapid warming.

Taxon & Cultivar	NCGR Local no.	4-week regrowth %			
		Encapsulation	Osmotic dehydration	Air dehydration	LN
Blackberry					
R. caesius (wild)	28.001	100	100	80	80
R. caucasicus (seedling selection)	54.001	100	100	80	80
R. cissoides Bush Lawyer (wild)	772.001	100	100	100	80
R. drejeri (wild)	55.002	100	100	100	100
R. hybrid 'Cherokee'	67.001	100	100	80	80
R. hybrid 'Dirksen Thornless'	71.001	100	100	80	95
R. hybrid 'Ebony King'	73.001	100	100	80	95
R. hybrid 'Olallie'	76.001	100	100	100	90
R. hybrid 'Ashton Cross'	317.001	100	100	100	90
R. hybrid OSU & USDA selection	344.002	100	100	100	90
R. hybrid 'Chester Thornless'	839.001	100	100	80	90
R. hybrid 'Loch Ness'	1863.001	100	100	80	70
Raspberry					
R. hybrid 'Black Diamond'	2229.001	100	100	100	95
R. idaeus 'Skeena'	124.001	100	100	80	60
R. idaeus 'Washington'	130.001	100	100	100	100
R. idaeus 'Willamette'	386.001	100	100	100	80
R. idaeus 'Comet'	1165.001	100	100	100	100
R. occidentalis 'Munger'	490.001	100	100	100	75

For LN exposure n = 20 and for each of the pretreatment controls n = 5.

Vitrification

Cold acclimation: Shoot tips of 'Burbank Thornless' exposed to PVS2 but not to LN showed distinct improvement in regrowth with cold acclimation, with regrowth ranging from 60% at one week to 100% at 3 and 4 weeks. LN-exposed shoot tips were less resilient and reached only 22% regrowth with 4-week cold acclimation. Cold acclimation of 4 weeks was significantly better than 1-3 weeks. Fewer than 10% of the shoot tips from 1-3 week cold acclimated plants regrew. Thus for further experiments plants were cold acclimated for 4 weeks.

Exposure to PVS2: All of 'Burbank Thornless' shoot tips grew following LS treatment on ice for 15 to 40 min. Subsequent exposure to PVS2 on ice severely reduced regrowth of shoot tips. LN exposure reduced recovery even more with 20 min PVS2 exposure producing significantly more ($P \leq 0.05$) living shoots than other exposure times (Fig. 4).

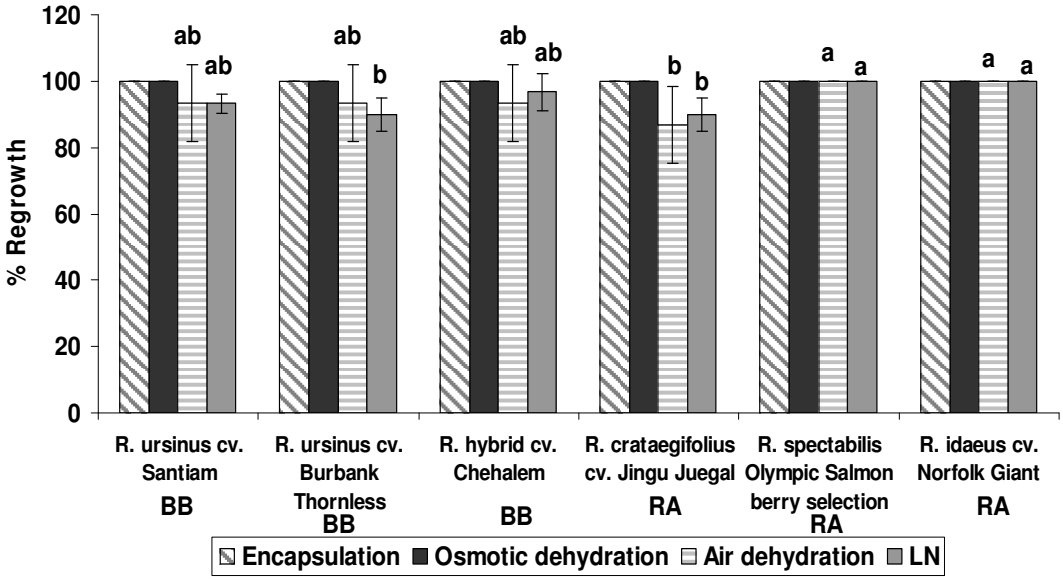

Figure 3: Genotype response of *Rubus* accessions cryopreserved by encapsulation-dehydration. Data are mean ± SD. (BB = blackberry, RA = raspberry). Means within a treatment followed by different letters are significantly different at $P \leq 0.05$.

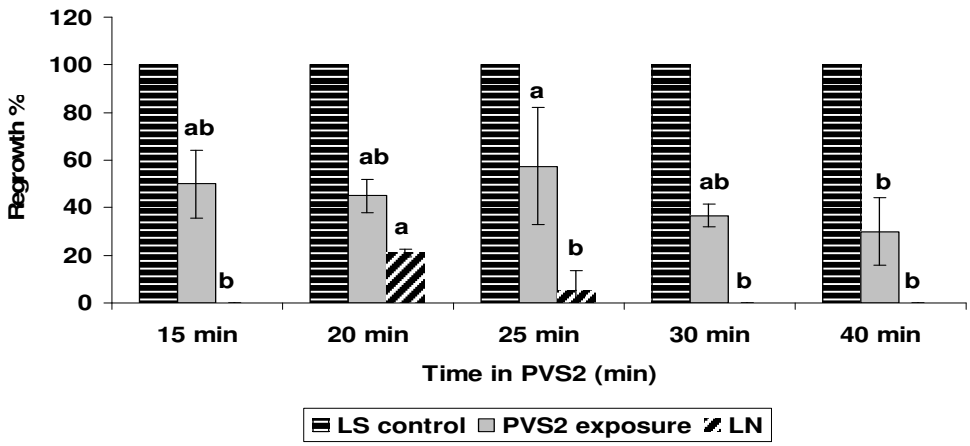

Figure 4: Regrowth of shoot apices of *R. ursinus* cv. Burbank Thornless following 5 PVS2 exposure times and LN. Shoot tips were treated with LS for 20 min on ice and PVS2 on ice and plunged in LN. Data are means ± SD. Means in a treatment followed by different letters are significantly different at $P \leq 0.05$.

Temperature of PVS2: Shoot tips treated with LS alone, either on ice or at room temperature, had 100% regrowth. Exposure to LS followed by PVS2 at ambient temperature resulted in about 65% regrowth following LN exposure (Fig. 5). Shoot tips treated with PVS2 at 0 ºC had significantly lower regrowth, ranging from 20 to 35%. Further experiments were performed at room temperature.

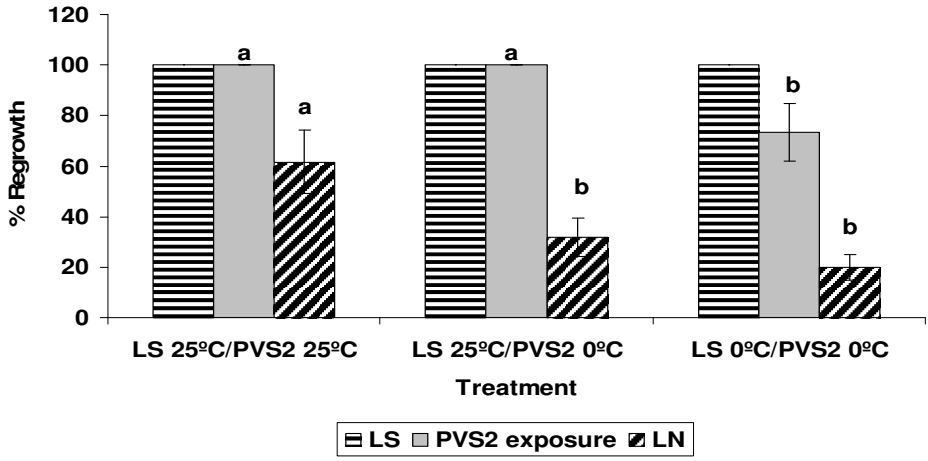

Figure 5: Regrowth of shoot apices of *R. ursinus* cv. Burbank Thornless cryopreserved using vitrification protocol. Shoot tips were treated with LS and PVS2 for 20 min at RT or on ice then plunged into LN. Data are means ± SD. Means in an exposure category followed by different letters are significantly different at $P \leq 0.05$.

Genotype response: Clear genotypic differences were observed in shoot tip regrowth among the four genotypes treated with the optimized procedure (Fig. 6). 'Burbank Thornless,' 'Jingu Juegal,' and 'Chehalem' had significantly better shoot growth (65-78%) than 'Hull Thornless' (45%). Evidence of regrowth was apparent by the second week after thawing for all four cultivars, rising to the maximum by week 4. All shoot tips grew into shoots without undergoing a callus phase.

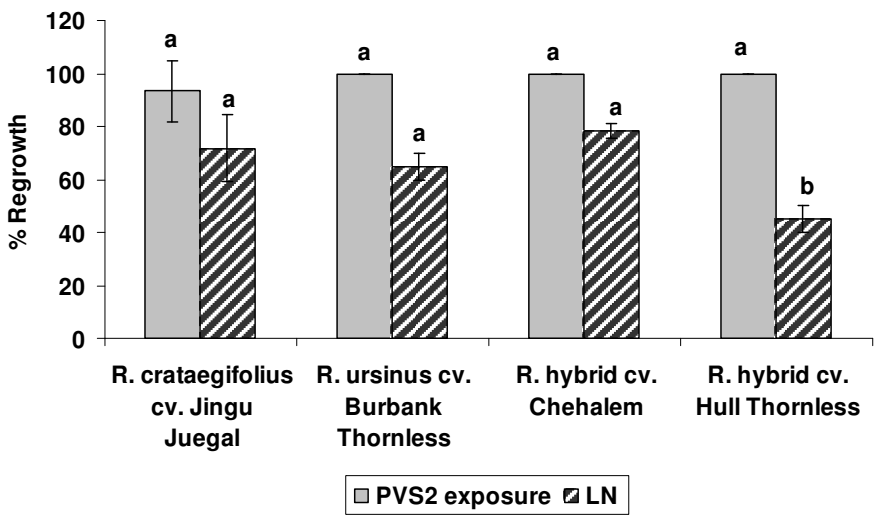

Figure 6: Regrowth of shoot apices of *Rubus* spp. cryopreserved using the vitrification protocol. Shoot tips were treated with LS and PVS2 for 20 min each at room temperature and plunged into LN. Data are means ± SD. Means in a treatment followed by different letters are significantly different at $P \leq 0.05$.

Comparison of encapsulation-dehydration and vitrification protocols

'Burbank Thornless' and 'Chehalem' responded significantly better to encapsulation-dehydration than to vitrification (Fig. 7). Differences were not significant for 'Jingu Juegal.' PVS2-vitrified shoots emerged at 2 weeks compared to 1 week with E-D. Both protocols provided recovery suitable for storage of germplasm.

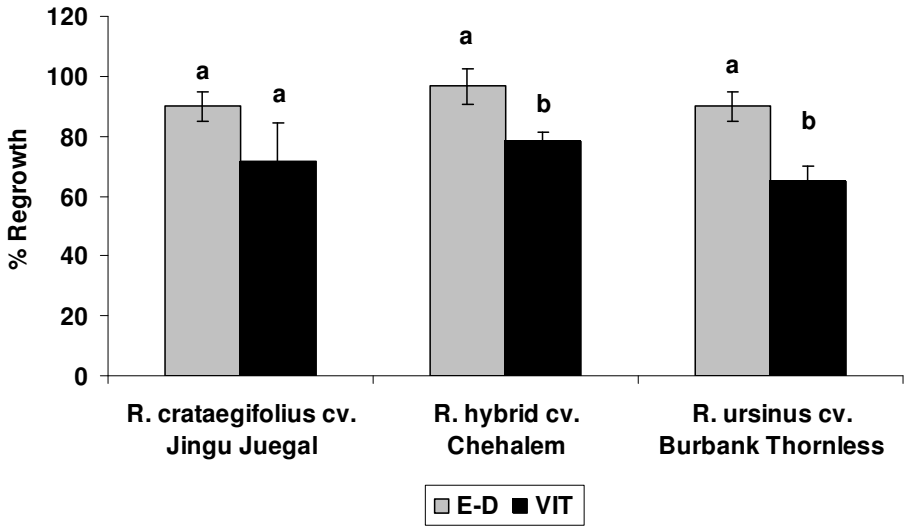

Figure 7: Regrowth of shoot apices of *Rubus* spp. cryopreserved using the optimized encapsulation-dehydration and vitrification protocols. Data are means ± SD. Means in a genotype followed by different letters are significantly different at $P \leq 0.05$.

DISCUSSION

All blackberry and raspberry genotypes investigated regrew following cold acclimation, preculture steps, dehydration and LN exposure. Cold acclimation was essential for successful cryopreservation by both E-D and vitrification (Fig. 1). Cold-acclimated plants had increased dehydration resistance and recovered well following all pretreatment steps and LN exposure. Shoot tips with less than optimum cold acclimation (less than 3 weeks) had either low recovery or did not survive dehydration and subsequent LN exposure. The importance of the amount and regime of cold acclimation in *Rubus* was demonstrated earlier, showing that 1-week cold acclimation doubled regrowth of four genotypes cryopreserved by slow freezing (18) and an alternating cold/warm cycle of acclimation was the most effective for inducing deep cold hardiness (7). For a slow freezing protocol, 2 weeks of cold acclimation were required for optimal regrowth (80%) of *R. parvifolius* while *R. caesius* required 10 weeks to reach 80% regrowth (6). In the current study, using the optimized encapsulation-dehydration protocol *R. caesius* had 80% regrowth after 4 weeks cold acclimation. The influence of cold acclimation in improving the efficiency of cryopreservation protocols is clearly demonstrated for many temperate fruit species in earlier studies using various methods: *Ribes* [Slow Freezing (SF), Vitrification (VIT), Encapsulation-Dehydration (E-D)], *Rubus* (SF), *Pyrus* (SF, VIT, E-D), *Malus* (E-D) and *Fragaria* (E-D, E-VIT) (4,6,8,9,12,16,23,24).

Encapsulation-dehydration

Recovery following encapsulation-dehydration is normally dependent on the amount of freezable water present in the cells (3, 5). The bead water content below 26% of fresh weight (0.4 g water g^{-1} dry weight) required for vitrification of cell water as noted by Block (5) was met at 4 h of air desiccation (Table 2). Freezable water within plant tissues encapsulated in calcium-alginate matrix was removed by both osmotic dehydration and air desiccation. The success of the method is largely dependent upon the plant's desiccation tolerance and the ability to circumvent ice nucleation during cooling and warming. Moisture content profiles (Fig. 2) show the correlation between moisture content of the tissues and recovery after cryopreservation. As optimized for cryopreserved *Rubus* apices, about 20% RM after dehydration was also best for apple (16) and pear (9) shoot apices. Wang et al. (29) tested an E-D procedure that included 0.4 M sucrose and 2M glycerol in 2.5% alginate beads on seven *Rubus idaeus* selections and obtained an average of 55% regrowth. The main difference between Wang et al., (29) and our E-D protocol was the composition of the alginate beads. The four *R. idaeus* cultivars in our study cryopreserved by E-D used beads with 3% alginate and 0.4 M sucrose, but no glycerol. The mean regrowth following air dehydration was 95% and 85% after LN exposure (Table 3).

The high regrowth obtained for E-D *Rubus* shoot tips indicates optimized cell moisture content. Differential scanning calorimeter analysis performed on encapsulated shoot tips dehydrated to low moisture contents showed a correlation among dehydration, the formation of the glassy state, and survival after cryopreservation (14). Ice nucleation was always associated with insufficient dehydration and mortality. Unlike the original E-D technique where encapsulated shoot tips are rewarmed gradually at ambient temperature (9), in this study the encapsulated shoot tips were rewarmed rapidly in 45ºC water for 1 min then at 25ºC. Shoot tips retained their capacity for shoot formation in all blackberry and raspberry cultivars. Rapid rewarming resulted in more regrowth than slower warming in garlic (13). A callus phase prior to shoot formation is highly undesirable since it may increase the frequency of somaclonal variation. *Rubus* cryopreserved shoot tips regrew without intermediary callus following recovery on medium without IBA, as noted earlier by Chang & Reed (6). All cultivars exhibited apparent regrowth within the first week after warming, rising to its maximum level by week 2. All survivors regenerated shoots. Genotypic differences using this technique were minimal ranging from 60 to 100% regrowth (Table 3).

Vitrification

PVS2 vitrification was successful in this study with a mean regrowth of 71% for the three blackberries tested compared to 92% for the same genotypes using E-D. There are no direct comparisons for this study; however encapsulation-vitrification (ED-Vit) studies of *R. idaeus* produced a mean of 75% regrowth for 7 genotypes and 68% for 4 other genotypes (29). The vitrification procedure requires highly concentrated solutions to dehydrate the cells without causing injury and produces a stable glass when plunged in LN (26). Vitrification solutions are potentially injurious to shoot tips due to the phytotoxic effects of individual chemical components or their combined osmotic effects on cell viability. It is essential that procedures are optimized for genotypic differences to dehydration and chemical sensitivity (21). The optimization of pretreatments, PVS2 exposure and regrowth protocols all contribute to the recovery of shoots following vitrification procedures. In this case 20 minutes in PVS2 at 25°C produced the best regrowth following exposure to LN; considerably less time compared to 3 h at 24°C or 5 h at 0°C required for Encapsulation -Vitrification (29).

Genotypic differences to LN exposure were reported in *Fragaria*, *Pyrus*, *Rubus* cultivars when using controlled freezing (21) and in *Ribes* when using controlled freezing, encapsulation-dehydration and vitrification (4). The optimized encapsulation-dehydration

protocol worked very well for both raspberry and blackberry fruit types and the vitrification protocol was successful for several species of blackberry. The genotypes tested were representative of the diversity in the genus *Rubus*, indicating that the protocol can be used to conserve a wide range of blackberry and raspberry genetic resources. From a practical perspective, these protocols can be routinely applied to diverse *Rubus* genotypes for the long-term storage of the *Rubus* germplasm maintained at NCGR (USDA-ARS) and NBPGR.

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